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(54) Title: PROTEIN CAPABLE OF SELF-ASSEMBLY AT A HYDROPHOBIC-HYDROPHILIC INTERFACE AND USES THEREOF

(57) Abstract: The invention relates to a hydrophobin-like protein capable of self-assembly at a hydrophobic-hydrophilic interface. The protein according to the present invention belongs to a new class of protein and is an at least partially purified protein comprising a polypeptide having at least 40 % identity and at least 5 % similarity with at least one polypeptide chosen from the group consisting of i) amino acids 29 - 131 of SEQ NO. 1 and ii) amino acids 29 - 133 of SEQ. NO. 2.

PROTEIN CAPABLE OF SELF-ASSEMBLY AT A HYDROPHOBIC-HYDROPHILIC INTERFACE AND USES THEREOF

The present invention relates to a protein capable of self-assembly at a hydrophobic-hydrophilic interface.

Such proteins are known in the art, and in particular hydrophobins have been disclosed. Hydrophobins are a well-defined class of proteins (Wessels et al, 1997) capable of coating a surface, rendering a hydrophobic surface hydrophilic, and often vice versa. They have a conserved sequence

$$X_n\text{-}C\text{-}X_{5\text{-}9}\text{-}C\text{-}C\text{-}X_{11\text{-}39}\text{-}C\text{-}X_{8\text{-}23}\text{-}C\text{-}X_{5\text{-}9}\text{-}C\text{-}C\text{-}X_{6\text{-}18}\text{-}C\text{-}X_m$$

X, of course, represents any amino acid, and n and m, of course, independently represent an integer. In general, a hydrophobin has a length of up to 125 amino acids. The cysteine residues (C) in the conserved sequence are part of disulphide bridges. These hydrophobins are typically isolated from fungi like Schizophyllum commune.

It is desirable to have the disposal of a varied range of proteins having the above property to accommodate the requirements for any particular application of said proteins, such as coating objects.

Therefore the present invention relates to a protein according to the preamble, characterized in that the protein is an at least partially purified protein comprising a polypeptide having at least 40% identity and at least 5% similarity to at least one polypeptide chosen from the group consisting of i) amino acids 29 - 131 of SEQ NO. 1 and ii) amino acids 29 - 133 of SEQ. NO. 2.

Thus, according to the present invention, a new class of hydrophobin-like proteins is provided differing markedly from known hydrophobins, in that the above-defined conserved sequence is not present.

In the present application, the term "identity" used in association with polypeptide is defined, in accordance with the state of the art, as having exactly matched amino acid residues. Here, sequences may comprise insertions or

deletions.

The term "similarity" used in association with polypeptide denotes conservative substitutions. Conservative substitutions are substitutions in which one amino acid is replaced with another, where the following amino acids are considered similar:

A,S,T;

D,E;

N,Q;

R,K;

I,L,M,V;

F,Y,W.

The protein may be derived from a filamentous bacterium, in particular a bacterium capable of forming aerial hyphae such as an Actinomycete, and more specifically the filamentous bacterium may be a Streptomyces species. A Streptomyces species from which the protein may be isolated using standard procedures for the isolation of hydrophobins, is a Streptomyces species which has been transformed with a construct that can be isolated from an E. coli strain which has been deposited on 14 March, 2000 under accession number CBS 102638 with the Centraalbureau voor Schimmelcultures (Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands).

Preferably, the identity is at least 50%, more preferably at least 60% and most preferably at least 70%.

Also, the similarity is at least 7%, preferably at least 10% and more preferably at least 15%.

It is thought that such proteins more specifically define proteins having better suitability for coating a surface. It goes without saying that, provided the polypeptide as defined above is present, the protein comprising said polypeptide may contain any additional sequences, unusual aminoacids, both with respect to stereo-chemistry as to structure, as long as the capability of self-assembly at a hydrophobic-hydrophilic interface is not compromised.

In accordance with the above, the present invention also relates to a method of coating a surface of an object, wherein a protein according to the present invention is used.

According to a preferred embodiment, the object is chosen from the group consisting of a window, a contact lens, a biosensor, a medical device, a container used for an assay or storage, the hull of a vessel (ship), or a frame or body-work of a car, and a solid particle, whereby a solution comprising the protein according to the invention is contacted with said object.

If it is desired to change the hydrophobic/hydrophilic nature of a surface, a hydrophilic surface is coated with an amount of protein sufficient to provide the coated surface of the object with a contact angle for water larger than 60°, and a hydrophobic surface is coated with an amount of protein sufficient to provide the coated surface of the object with a contact angle for water smaller than 90°. It is also possible to use the coated surface to chemically attach various compounds, such as prosthetic groups, antibodies etc., to the protein according to the invention using methods well-known in the art.

The present invention also relates to a method of stabilizing a dispersion, such as an oil or fat in water-dispersion, wherein a protein according to the invention is used to stabilize the dispersed particles. The stabilized particles may also be solid particles, such as latex spheres suitable for assays, such as immuno-assays.

It is thought that a treatment at a temperature between 30° and 90°C, optionally in the presence of a surfactant and as disclosed in PCT/NL01/00084, the description of which is incorporated by reference, may induce a conformational change resulting in a substantially irreversible change in the structure of the protein, as a result of which a coating is rendered insoluble.

In contrast to known hydrophobins, the proteins according to the invention isolated until now by the present inventors, have only up to one disulphide bridge. This may be advantageous for particular applications. Should this be desirable or necessary, it is thought that the hydrophobin may be stabilized as disclosed in PCT/NL01/00082, the description of which is included by reference.

The protein according to the invention may be iso-

lated, should this be desirable or necessary, using the method disclosed in PCT/NL01/00083, the description of which is included by reference.

The invention will now be illustrated with reference to the following examples and the drawing in which

fig. 1 depicts an SDS-PAA gel showing the protein according to the present invention;

fig. 2 depicts a Northern blot;

fig. 3 shows a picture of an immuno-gold labeling of RdlA and RdlB proteins at the outer surface of aeral hyphae; and

fig. 4 shows a picture indicating the difference in attachment of hyphae of wild-type Streptomyces lividans and a control strain (S. lividans) lacking RdlA and RdlB proteins to polystyrene, and a graph quantitatively showing this relative binding for both S. lividans and S. coelicolor.

PREPARATIONS

METHODS

Strains and plasmids

Cloning in *Escherichia coli* was done in DH5α or JM110. *Streptomyces coelicolor* A3(2) strain M145 and *Streptomyces lividans* TK23 (Kieser *et al.*, 2000) were used throughout this study, while *Streptomyces griseus* (DSMZ 40236) and *Streptomyces tendae* Tü 901/8c (Richter *et al.*, 1998) were used to establish whether other streptomycetes contain homologues of *rdlA* and *rdlB*. Vectors are summarised in Table 1.

GROWTH CONDITIONS AND MEDIA

Streptomyces strains were grown at 30 °C on solid MS agar medium or in YEME medium as liquid shaken cultures (Kieser *et al.*, 2000). Spores were harvested with water and stored as described previously (Kieser *et al.*, 2000). To assess attachment, *S. coelicolor* and *S. lividans* were grown in 96-well flat-bottomed microtiter plates (Costar, Corning Incorporated, USA) containing 200 µl NMMP (Kieser et al., 2000; in the absence of PEG6000 and using 50 mM glucose as a carbon source). Prior to inoculation, spores, stored at

-20 °C in 20% glycerol, were washed and diluted in medium to a final concentration of 5 · 10^6 spores ml^{-1}. 96 well flat-bottomed microtiter-plates were filled with 200 µl spore suspension per well.

TABLE 1.

Plasmids

Plasmid	Description	Reference
pGEM-T	Linear plasmid used for cloning PCR fragments amplified with TAQ polymerase in *E. coli*	Promega
pBluescript KS+	pUC18 derivative for cloning in *E. coli*	Stratagene
pZero-2.1		Invitrogen
pC46a	pBluescript KS+ containing a 4,5 kb *Sal*I fragment of cosmid 46 (Redenbach *et al.*, 1996) of *Streptomyces coelicolor* encompassing the coding sequences of *rdlA* and *rdlB*, their interspersed promoter region and flanking regions of 0,8 kb (*rdlA*) and 2,5 kb (*rdlB*)	This work
pC46b	pZero2.1 containing the *Sal*I fragment described for pC46a	This work
pC46c	pC46b derivative carrying a 1,4 kb *Sma*I fragment containing a hygromycine resitance cassette (Zalacaín *et al.*, 1986) replacing a 0,8 kb *Blp*I / *Sca*I fragment encompassing *rdlA*, the 5' end of the coding sequence of *rdlB* as well as their interspersed promoter region	This work
pC46d	pC46c derivative carrying a 1.8 kb apramycin resistance cassette contained on a *Sma*I fragment (Prentki and Krisch, 1984) cloned in the XbaI site of pC46c	This work

MOLECULAR TECHNIQUES

Standard molecular techniques followed Sambrook *et al.* (1989). Protoplast preparation and transformation were performed as described by Kieser *et al.* (2000) using alkali-denatured DNA (Oh and Chater, 1997). The rich solid medium R2YE was used for regenerating protoplasts overlaid with the appropriate antibiotic after 18 hrs. Chromosomal DNA of *S. coelicolor* and *S. lividans* was isolated according to Verhasselt *et al.*, (1989) and modified by Nagy *et al.* (1995). Total RNA of *S. coelicolor* and *S. lividans* was isolated using the SV Total RNA Isolation System (Promega). DNA and RNA were blotted on Nylon filters (Boehringer, Mannheim) and

hybridised under conditions described by Church and Gilbert (1984) at 60 °C. Under these conditions *rdlA* and *rdlB* do not cross-hybridise. Radioactively labelled probes were made using the oligolabelling kit (Pharmacia, Uppsala).

ISOLATION OF THE *rdlA* AND *rdlB* GENES FROM *S. coelicolor* A3(2) AND *S. lividans* TK23

To isolate *rdl* genes from *S. coelicolor* A3(2) and *S. lividans*, a degenerate oligonucleotide (SGCSGASAGSACSGASAGGTCCTCSAGSACGTGSGASAGSGCGCCGTC) representing the N-terminal sequence of an internal peptide of RdlA of *S. lividans* (see Results) was radioactively labelled and hybridised to the cosmid library of *S. coelicolor* A3(2) (Redenbach *et al.*, 1996).

CONSTRUCTION OF THE *rdlAB* GENE DELETION PLASMID

The *rdl* genes were deleted by replacing a 0.8 kb *Blp*I/*Sca*I fragment of pC46b (see Table 1) containing the entire coding sequence of *rdlA* and 136 bp of *rdlB* as well as the interspersed promoter region by a 1.4 kb *Sma*I fragment containing the hygromycin B resistance gene (Zalacaín *et al.*, 1986). This resulted in vector pC46c. A 1.8 kb *Sma*I fragment containing the apramycin resistance gene was cloned in the *Xba*I site of pC46c to select for double crossover events, resulting in plasmid pC46d.

PREPARATION OF CELL WALLS AND PROTEIN EXTRACTS

Filaments of *S. coelicolor* A3(2) and *S. lividans* were fragmented at 20,000 psi using a SLM French® Pressure Cell Press. Cell walls were treated with 2% SDS at 100 °C as described (Wessels *et al.*, 1991a) (Wessels *et al.*, 1991b) and subsequently extracted with TFA (Wösten *et al.*, 1993). After removal of the solvent by a stream of air, extracts were taken up in SDS sample buffer (2% SDS, 20% glycerol, 0.02% bromophenolblue, 0.1 M Tris-HCl, pH 6.8) and subjected to SDS-PAGE. If necessary, adjustments of pH were done by addition of 25% ammonia. RdlA and RdlB were purified by taking up TFA extracts of SDS-treated cell walls in water without shaking. Insolubles were removed by centrifugation at 10,000 g for 15 minutes.

ELECTROPHORESIS AND WESTERN BLOTTING

SDS-PAGE was done in 16% SDS-polyacrylamide gels. Prestained broad range molecular weight markers of Biorad were used. After separation, proteins were stained with 0.25% Coomassie Brilliant Blue G-250 (CBB) or blotted onto a polyvinylidenedifluoride (PVDF) membrane using semi-dry blotting. For N-terminal sequencing, a PVDF membrane was stained with CBB and a slice of the membrane containing the protein was excised. After destaining with 30% methanol, the N-terminal sequence was determined using a pulse liquid sequenator on line connected to a PTH analyser (Eurosequence, Groningen, The Netherlands). To determine N-terminal sequences of internal peptides, the protein was eluted from the SDS-PAA gel followed by a tryptic digestion. Peptides were sequenced after separation on a C18 reversed phase HPLC column.

Antibodies against RdlA and RdlB were raised by injecting a mixture of these proteins of *S. lividans* eluted from a SDS-PAA gel. PVDF membranes were treated with diluted anti-RdlA/RdlB serum (1:1000) as described (Harlow and Lane, 1988).

IMMUNOLOCALIZATION

Fixation, embedding and immuno-labelling of cultures were performed as described (Wösten *et al.*, 1994) with the modification that K4M was substituted for Unicryl. Polyclonal antibodies raised against a mixture of RdlA and RdlB of *S. lividans* were purified with an acetone powder of mycelium(Harlow *et al.*, 1988) of a liquid shaken culture and diluted 1000 times.

ATTACHMENT ASSAY

To quantify attachment of *S. coelicolor* and *S. lividans* 25 µl 0.5% crystal violet (Acros Organics) was added to each well and incubated for 10 minutes to stain cell material. This was followed by washing three times with 200 µl of water using a Vaccu-Pette/96 (Sigma), removing all non-adherent cells. After drying overnight at 30 °C the adherence of cells was quantified by solubilizing the crystal violet

with 200 µl of 10% SDS (Reynolds and Fink, 2001) during 30 minutes under shaking conditions (900 rpm). 100 µl was transferred to a new well and the OD_{570} was determined using a microtiter plate reader. If necessary, dilutions were made in 10% SDS. Total biomass formed was determined using Lowry with the modification that cells were treated at 100 °C for 30 minutes in 0.2% SDS/1 M NaOH. BSA was used as a standard. The relative attachment was determined by comparison of the OD_{570} of wildtype and disruptant strains.

RESULTS

Identification of an abundant SDS-insoluble cell wall protein specifically present in aerial structures of *S. coelicolor* and *S. lividans*

Cell walls of a 5-day-old sporulating culture of *Streptomyces lividans* grown on solid MS medium were treated with 2% SDS at 100 °C. After washing with water and lyophilizing, this was followed by an extraction with trifluoroacetic acid (TFA). SDS-PAGE of the SDS-soluble fraction showed a complex pattern of polypeptides (results not shown). Among the proteins that were insoluble in hot SDS but soluble in TFA, an abundant polypeptide, called Rdl, was observed with an apparent molecular weight of 18 kDa (Figure 1, left panel). Western analysis with antibodies raised against this protein showed the absence of it in a TFA extract of SDS-treated walls of liquid shaken cultures and in the SDS-soluble fractions of shaken and solid cultures (results not shown). In addition, it was shown that the presence of Rdl correlated with the presence of aerial hyphae. In cell walls of 1-day-old cultures not yet forming aerial hyphae Rdl was absent (data not shown). In contrast, the protein was abundantly present in cultures that had formed a confluent layer of aerial hyphae.

When water instead of 2% SDS was added to the TFA extract of SDS-treated cell walls of cultures forming aerial hyphae, Rdl was the main protein that dissolved (Figure 1, right panel). In water the extract formed an SDS-insoluble complex upon shaking that could be dissociated with TFA. Similar results as obtained with *S. lividans* were obtained

with cultures of *S. coelicolor*. These data indicate that, under physiological conditions, Rdl is an insoluble cell wall protein specifically present in cultures of *S. lividans* and *S. coelicolor* forming aerial structures.

CLONING AND CHARACTERIZATION OF THE *rdl* GENES OF *S. lividans* AND *S. coelicolor*

N-terminal sequencing revealed that the Rdl protein of *S. lividans* running at the 18 kDa position was in fact a mixture of two similar proteins, called RdlA and RdlB, with slightly different N-termini (Seq. 1 starting at amino acid 29 and Seq. 2 starting at amino acid 29). In addition, N-termini of two internal peptides were determined that resulted from a tryptic digestion of a mixture of RdlA and RdlB. A radioactive degenerated oligonucleotide based on one of the peptides was used to screen a cosmid library of *S. coelicolor* A3(2) (Redenbach *et al.*, 1996). The oligonucleotide hybridised to the overlapping cosmids C46 and C61. The hybridising fragment of cosmid C46 was contained on a 4.5 kb SalI fragment. This fragment was cloned in pBluescript KS+ in the unique SalI site, and introduced in E. coli DH5a (deposited on 14 March, 2000 under accession number CBS 102638 with Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands) and partially sequenced. An ORF was identified that encodes a polypeptide of 131 aa. It starts with a putative signal sequence for secretion followed by a sequence corresponding to the determined N-terminal sequence of RdlA as found in the cell wall (mature RdlA). The N-terminal sequences of both internal peptides were also identified in the ORF. The *rdlB* gene (Seq. 4), divergently transcribed from *rdlA* (Seq. 3), was identified 262 bp upstream of the start codon of *rdlA*. It encodes a protein very similar to that encoded by *rdlA* (68.7% identity, 14.5% similarity) and contains the N-terminus of mature RdlB preceded by a putative signal sequence of 28 amino acids. Sequences 3 and 4 represent the coding sequences of *rdlA* and *rdlB* including the leader or signal peptide of 28 amino acids (starting at GTG).

The coding sequences of *rdlA* and *rdlB* hybridised to

the same unique fragments of genomic DNA of *S. coelicolor* and *S. lividans* digested with a variety of enzymes (one at a time). For instance, a 4.5 kb *Sal*I fragment hybridised to both *rdlA* and *rdlB*. A slightly larger genomic fragment hybridised after digestion with *Blp*I, while digestion with *Pst*I resulted in a fragment of about 8 kb (data not shown). The almost complete genome sequence of *S. coelicolor* (http://www.sanger.ac.uk/Projects/S_coelicolor/) did not reveal homologous sequences of *rdlA* and *rdlB*.

Using PCR and primers based on the gene sequences of *rdlA* and *rdlB* of *S. coelicolor*, the sequences of the homologues of *S. lividans* were isolated and appeared to be identical to the sequences of *S. coelicolor*. Digested genomic DNA of *Streptomyces tendae* and *Streptomyces griseus* hybridized with a probe directed against the coding sequence of *rdlA* (data not shown). In *S. tendae* two fragments of 4.2 and 2.8 kb hybridized while in *S. griseus* fragments of 3.1, 2.2 and 1.0 kb reacted. Hybridization with *rdlB* showed that also homologues of this gene are present in these streptomycetes (data not shown).

rdlA AND *rdlB* ARE EXPRESSED IN AERIAL HYPHAE

To determine the temporal expression of *rdlA* and *rdlB* total RNA was isolated from cultures of *S. coelicolor* and *S. lividans* grown in liquid YEME medium or on MS plates. After separation on a formaldehyde gel and blotting to a Nylon membrane, RNA was hybridised with a probe representing the coding sequence of *rdlA* or *rdlB* (Figure 2). Expression of both *rdl* genes correlated with the formation of aerial hyphae. *rdl* mRNA was not detected in 1-day-old cultures (lower panel lane 1) not yet forming aerial hyphae, nor in 3, 4, and 7-day-old sporulating cultures (lower panel, lanes 3-5). However, it accumulated to high levels in 2-day-old cultures forming aerial hyphae (lower panel, lane 2). To verify that equal amounts of RNA were loaded in each lane of the gel, the Northern was hybridized with a probe representing 16S rRNA (upper panel). Accumulation of *rdl* mRNA was not observed in liquid shaken cultures (not shown).

RdlA AND RdlB ARE LOCALIZED AT THE OUTER SURFACE OF

AERIAL HYPHAE AND SPORES

RdlA and RdlB were localized using an antiserum raised against a mixture of RdlA and RdlB of *S. lividans*. In cross sections of cultures of *S. lividans* and *S. coelicolor* grown on solid MS medium immuno-gold-labelling was observed at the outer surface of cell walls of aerial hyphae (Figure 3) and spores, but not of submerged hyphae (data not shown). The reactive layer at the outside of the wall was sometimes detached, indicating that it is a discrete layer. The antiserum did not react with cultures not forming aerial hyphae, i.e. cultures of *S. lividans* and *S. coelicolor* grown in liquid shaken medium, and 1-day-old cultures grown on solid medium.

DISRUPTION OF *rdlA* AND *rdlB* DOES NOT AFFECT FORMATION OF AERIAL HYPHAE

The *rdlA* and *rdlB* genes were deleted in *Streptomyces coelicolor* and *Streptomyces lividans* with the deletion construct pC46d (see Methods). Gene replacement was confirmed by Southern analysis. A genomic 4.5 kb *Sal*I fragment of the wildtype strains hybridizes to probes against *rdlA* and *rdlB*. In the mutant strains an expected 3.2 kb fragment hybridized with a probe against *rdlB* while no hybridization was observed, as expected, with a probe against *rdlA* (data not shown). Germination, growth rates and differentiation of aerial hyphae into spores of wildtype and mutant strains were similar in different media and culture conditions (data not shown). In addition, the characteristic rodlet layer was observed at surfaces of aerial hyphae and spores in both the wildtype and the mutant strains (a.k.a. disruptants, which lack the *rdl* genes).

DISRUPTION OF THE *rdl* GENES AFFECTS ATTACHMENT OF HYPHAE TO POLYSTYRENE

Since RdlA and RdlB form a discrete surface layer on aerial structures (i.e. structures formed in a hydrophobic environment in this case the air), we tested disruptants of *S. coelicolor* and *S. lividans* for their capacity to adhere to the hydrophobic surface of standard 96 well microtiter plates of polystyrene. Strains were grown in liquid medium in the 96

well plates and attachment of hyphae was visualized with crystal violet (Figure 4 Upper part). Attachment of the mutant strains *S. coelicolor* ΔAB6 and *S. lividans* ΔAB3 was only 50-80% throughout culturing compared to that of the wildtype strains (Figure 4, graph). Reintroduction of *rdlA* in the *S. coelicolor* disruptant strain was sufficient to restore attachment to the hydrophobic solid (not shown).

EXPRESSION OF Rdl PROTEINS

As desired, the genes coding for the protein according to the invention may be introduced into a vector which is used to transform a host suitable for obtaining the protein in good yield and allowing for easy purification with the desired purity.

It is anticipated that for hosts excreting the protein according to the present invention even culture medium may be used to coat a surface. By eliminating the protein-producing organism, the supernatant qualifies as containing at least partially purified protein.

TEFLON CAN BE MADE WETTABLE USING RdlA AND RdlB

When a piece of Teflon was incubated overnight in the water-soluble fraction of the TFA extract of SDS-treated cell walls of *S. lividans* or *S. coelicolor* isolated from cultures forming aerial hyphae (and thus containing RdlA and RdlB (40 μg/ml); Figure 1, right lane), the Teflon became wettable showing that the protein coats the Teflon. This demonstrates the applicability of the protein for coating surfaces.

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SEQUENCE LISTING

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35 40 45

Asn Ser Met Thr Thr Gly Asn Met Ser Pro Gln Met Ala Leu Ile Gln
50 55 60

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Val Ile Gly Leu Val Pro Ile Gln Asp Leu Asn Val Leu Gly Asp Asp
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Met Asn Gln Gln Cys Ala Glu Asn Ser Thr Gln Ala Lys Arg Asp Gly
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Glu Gly Gly Lys Gly
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tgcgccgaga actcgacgca ggccaagcgc gacggtgcgc tggcccacct cctggaggac 360
gtctcgatcc tgtcctccaa cggcgagggc ggcaagggct ga 402

CLAIMS

1. Protein capable of self-assembly at a hydrophobic-hydrophilic interface, **characterized** in that the protein is an at least partially purified protein comprising a polypeptide having at least 40% identity and at least 5% similarity with at least one polypeptide chosen from the group consisting of i) amino acids 29 - 131 of SEQ NO. 1 and ii) amino acids 29 - 133 of SEQ. NO. 2.

2. Protein according to claim 1, **characterized** in that the protein is derived from a filamentous bacterium.

3. Protein according to claim 2, **characterized** in that the filamentous bacterium is an aerial hyphae forming bacterium.

4. Protein according to claim 2 or 3, **characterized** in that the filamentous bacterium is an Actinomycete.

5. Protein according to claim 4, **characterized** in that the filamentous bacterium is a Streptomyces species.

6. Protein according to claim 5, **characterized** in that the Streptomyces species is transformed with a construct that can be isolated from an E. coli strain which has been deposited on 14 March, 2000 under accession number CBS 102638 with the Centraalbureau voor Schimmelcultures (Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, the Netherlands).

7. Protein according to any of the preceding claims, **characterized** in that the identity is at least 50%, preferably at least 60% and more preferably at least 70%.

8. Protein according to any of the preceding claims, **characterized** in that the similarity is at least 7%, preferably at least 10% and more preferably at least 15%.

9. Method of coating a surface of an object, **characterized** in that a protein according to any of the claims 1 to 8 is used.

10. Method of coating a surface of an object according to claim 9, **characterized** in that the object is chosen from the group consisting of a window, a contact lens, a biosensor, a medical device, a container for performing an

assay or storage, the hull of a vessel or a frame or bodywork of a car, and a solid particle wherein a solution comprising the protein according to any of the claims 1 to 8 is contacted with said object.

11. Method of stabilizing a dispersion, **characterized** in that a protein according to any of the claims 1 to 8 is used to stabilize the dispersed particles.

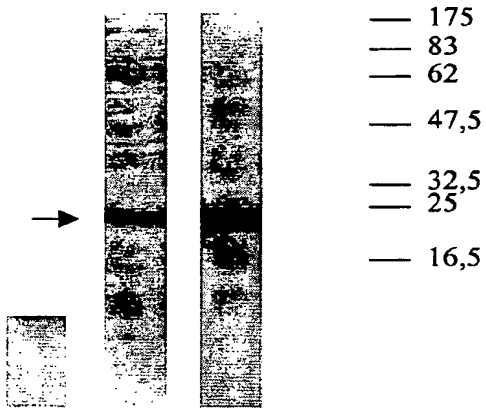


Fig. 1

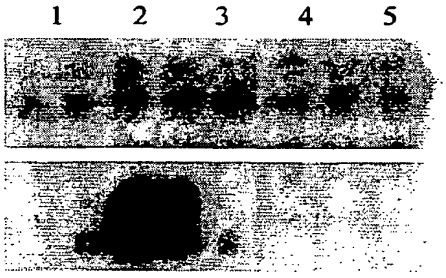


Fig. 2

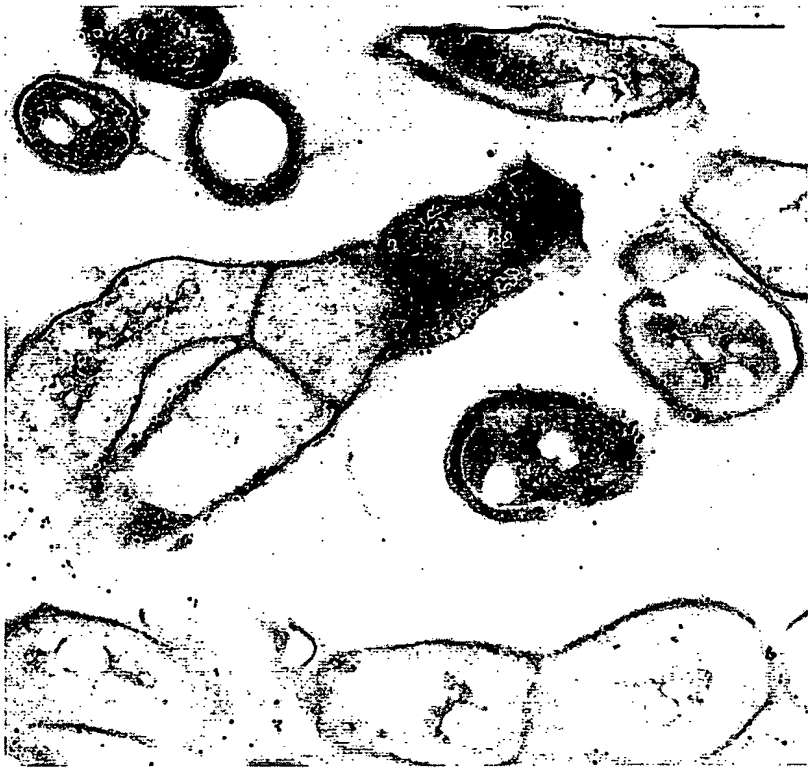
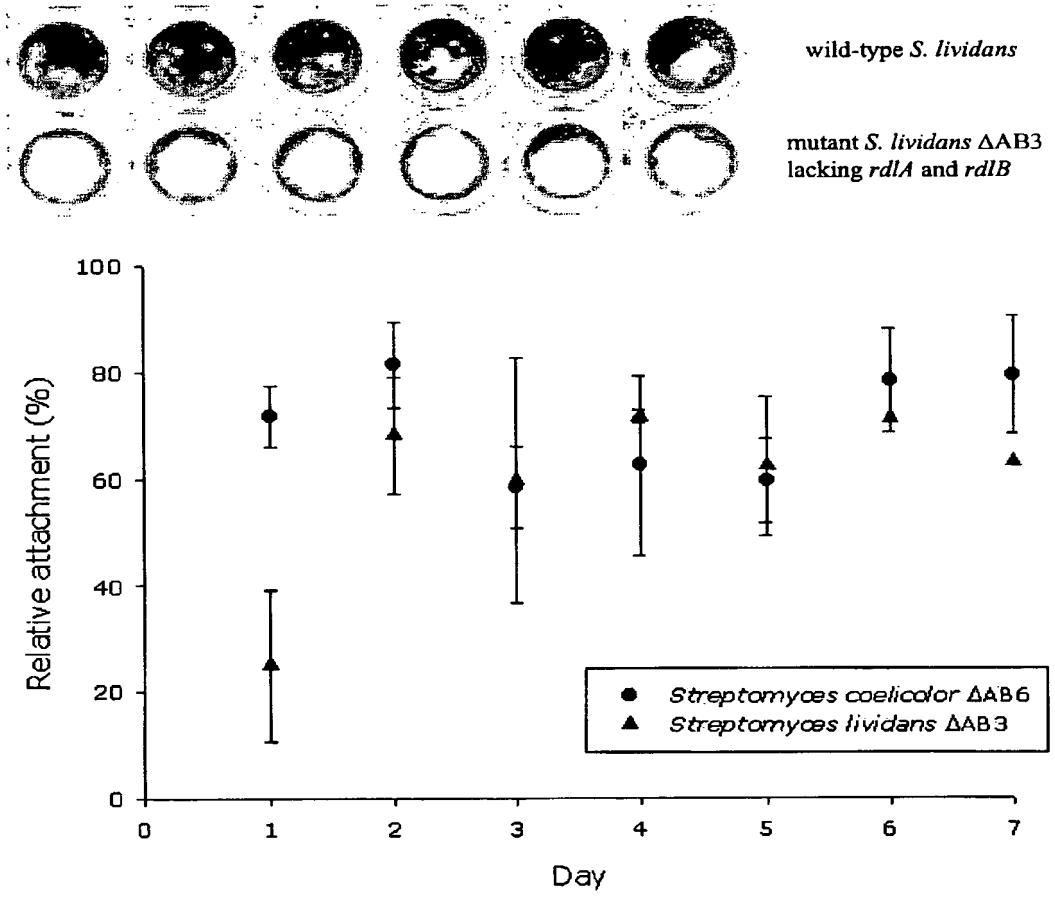


Fig. 3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mutant S. lividans ΔAB3 lacking rdlA and rdlB
Relative attachment (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Day
Streptomyces coelicolor ΔAB6
Streptomyces lividans ΔAB3

Fig. 4

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

STATEMENT IN THE CASE OF AN ORIGINAL DEPOSIT
pursuant to Rule 6.1

Centraalbureau voor Schimmelcultures
Oosterstraat 1
P.O. Box 273
3740 AG BAARN

name and address of
International Depositary Authority

THE UNDERSIGNED HEREBY DEPOSITS UNDER THE BUDAPEST TREATY THE MICROORGANISM IDENTIFIED HEREUNDER AND UNDERTAKES NOT TO WITHDRAW THE DEPOSIT FOR THE PERIOD SPECIFIED IN RULE 9.1[1]

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference[2]: C46a	☐ Mixture of microorganisms (Mark with a cross where applicable)
II. CONDITIONS FOR CULTIVATION	☐[3]
LB medium with 100 µg/ml ampicilline	

[1] This Form may also be used if the undersigned converts into a deposit under the Budapest Treaty the deposit of a microorganism that he or his predecessor in title has already deposited, outside the Budapest Treaty, with the same depositary institution either before (Rule 6.4(d)) or after the acquisition by that institution of the status of international depositary authority.
[2] Number, symbols, etc., given to the microorganism by the depositor.
[3] Mark with a cross if additional information is given on an attached sheet.

BP/A/II/12
page 5

III. CONDITIONS FOR STORAGE [3]
after growth in liquid medium, add glycerol to 15% and store at −80 °C

IV. CONDITIONS FOR TESTING VIABILITY [3]
Grow in LB medium with 100 µg/ml Ampicilline

V. COMPONENTS OF MIXTURE (where applicable) [3]
Description of components:
Method(s) for checking presence of components:

[3] Mark with a cross if additional information is given on an attached sheet.

VI. PROPERTIES DANGEROUS TO HEALTH OR ENVIRONMENT

☐ [4] The microorganism identified under I above has the following properties which are or may be dangerous to health or the environment: ☐ [3]

☒ [4] The undersigned is not aware of such properties.

VII. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION [5] ☐ [3]

Scientific description: E coli DH5α strain containing a pBluescript vector in which a 5 kb SalI fragment has been cloned. The 5 kb SalI fragment is derived from Streptomyces coelicolor A3(2) and contains the genes RdlA and RdlB

Proposed taxonomic designation:

VIII. ADDITIONAL DATA ☐ [6]

IX. DEPOSITOR

Name: L Dijkhuizen

Address: Dept Microbial
University of Groningen
Kerklaan 30
9751 NN Haren (NL)

Signature: [7]

Date: 13/3/2000

[3] Mark with a cross if additional information is given on an attached sheet.

[4] Mark with a cross the applicable box.

[5] It is strongly recommended that the scientific description and/or proposed taxonomic designation of the microorganism be indicated.

[6] Mark with a cross if additional information (other than the information referred to in footnote 3) is given on an attached sheet, such as the source of the microorganism, the name(s) and address(es) of any other depositary institution(s) with which the microorganism has been deposited, or the criterion used when drafting the proposed taxonomic designation. (The supplying of such information is optional)

[7] Where the signature is required on behalf of a legal entity, the typewritten name(s) of the natural person(s) signing on behalf of the legal entity should accompany the signature(s).

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

University of Groningen
Department of Microbiology
Kerklaan 30
9751 NN HAREN
Nederland

name and address of depositor

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
C46a	CBS 102638

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[ ] a proposed taxonomic designation

(mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary accepts the microorganism identified under I above, which received by it on 14-03-2000 *(date dd-mm-yy of the original deposit)* 1

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on not applicable *(date dd-mm-yy of the original deposit)* and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on not applicable *(date dd-mm-yy of receipt of request for conversion)*

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Centraalbureau voor Schimmelcultures

Address: Oosterstraat 1
P.O. Box 273
3740 AG BAARN
The Netherlands

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Mrs F.B. Snippe-Claus Dr J.A. Stalpers

Date (dd-mm-yy): 16-03-2000

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

University of Groningen
Department of Microbiology
Kerklaan 30
9751 NN HAREN
Nederland

name and address of the party to whom the viability statement is issued

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: University of Groningen Department of Microbiology Address: Kerklaan 30 9751 NN HAREN Nederland	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: CBS 102638 Date (dd-mm-yy) of the deposit or of the transfer: 1 14-03-2000

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 15-03-2000 [2]. On that date (dd-mm-yy), the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY HAS BEEN PERFORMED [4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Centraalbureau voor Schimmelcultures Address: Oosterstraat 1 P.O. Box 273 3740 AG BAARN The Netherlands	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Mrs F.B. Snippe-Claus Dr J.A. Stalpers Date (dd-mm-yy): 16-03-2000

[4] Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

Ir ional Application No
PCT/NL 01/00268

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/36 C09J189/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C09J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! Entry SCC46, 10 February 2000 (2000-02-10) BROWN S.P. ET AL.: "Streptomyces coelicolor cosmid C46" Database accession no. AL139164 XP002173536 the whole document & M. REDENBACH ET AL.: "A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb Streptomyces coelicolor A3(2) chromosome" MOLECULAR MICROBIOLOGY, vol. 21, no. 1, 1996, page 77 XP000876690 --- -/--	1-8

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 2001	14/08/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 01/00268

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WESSELS J G H: "FUNGAL HYDROPHOBINS: PROTEINS THAT FUNCTION AT AN INTERFACE" TRENDS IN PLANT SCIENCE,GB,ELSEVIER SCIENCE, OXFORD, vol. 1, no. 1, January 1996 (1996-01), pages 9-15, XP000866041 ISSN: 1360-1385 the whole document	1-11
A	R.D. TILLOTSON ET AL.: "A surface active protein involved in aerial hyphae formation in the filamentous fungus Schizophillum commune restores the capacity of a bald mutant of the filamentous bacterium Streptomyces coelicolor to erect aerial structures" MOLECULAR MICROBIOLOGY, vol. 30, no. 3, 1998, pages 595-602, XP002173534 the whole document	1-10
T	HAN A.B. WOSTEN ET AL.: "Surface-active proteins enable microbial aerial hyphae to grow into the air" MICROBIOLOGY, 'Online! vol. 146, April 2000 (2000-04), pages 767-773, XP002173535 Retrieved from the Internet: <URL:http://www.eleves.ens.fr:8080/home/gliga/hydrophobins.html> 'retrieved on 2001-07-24! the whole document	1-11